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HAEMOLYMPH LIPIDS OF HEALTHY AND DISEASED JAPANESE BEETLE LARVAE

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Abstract—Extractable lipids from the haemolymph of healthy and diseased larvae of the Japanese beetle, *Popilliae japonica*, have been characterized and compared. Total lipid content of normal larvae was 5·7 to 7·2 mg/ml haemolymph. This value was reduced to 3·1 to 3·9 mg/ml in haemolymph from larvae infected with the milky disease organism, *Bacillus popilliae*. Neutral lipids accounted for 70 per cent of total lipids in both normal and diseased larvae haemolymph. Phospholipids accounted for 30 per cent of the total lipids. The neutral lipid fraction is composed of sterols, monoglycerides, free fatty acids, 1,2- and 1,3-diglycerides, triglycerides, sterol esters, and hydrocarbons. Haemolymph phospholipids are composed primarily of phosphatidic acid, phosphatidyl choline, and phosphatidyl ethanolamine. The fatty acid composition of both neutral lipid and phospholipid fractions is predominately C_{16} and C_{18} fatty acids. Significant quantities of hydrocarbons have been detected in the neutral lipids of haemolymph from both healthy and diseased larvae. Infection with *B. popilliae* resulted in a non-selective decrease of 45 per cent in neutral lipids and phospholipids in haemolymph from third instar larvae.

INTRODUCTION

THE MILKY disease organism, *Bacillis popilliae* (Dutky), grows and sporulates readily only in the haemolymph of Japanese beetle larvae (*Popilliae japonica* Newman). So far, it has not been possible to develop an *in vitro* system that permits economical production of spores required for a self-propagating biological control of *P. japonica*. An excellent review of the milky disease in *P. japonica* has been published by RHODES (1968), and the fastidious nature of *B. popilliae* is demonstrated by the tissue culture experiments of LÜTHY *et al.* (1970). Analyses of haemolymph constituents have been conducted to determine the chemical environment required for the sporulation process of *B. popilliae* (SHOTWELL *et al.*, 1963; SHOTWELL *et al.*, 1965; STUBBLEFIELD *et al.*, 1966; WEINER *et al.*, 1966; BENNETT *et al.*, 1968; BENNETT and SHOTWELL, 1970). Also, the nutritional requirements of *B. popilliae* have been studied (SYLVESTER and COSTILOW, 1964; COULTER and COSTILOW, 1970). The lipids present in vegetative cells of bacteria (including *B. popilliae*) related to insect diseases have been reported by BULLA *et al.* (1970).

This paper reports the alterations of lipid components in larvae haemolymph as a result of infection with *B. popilliae*.

MATERIALS AND METHODS

Larvae

Third instar *P. japonica* larvae were maintained and injected with *B. popilliae* spores or vegetative cells as described by PRIDHAM *et al.* (1964). Haemolymph was collected as outlined by SHOTWELL *et al.* (1963) from healthy larvae and from larvae that became milky on the seventh day after injection. Larvae were bled into graduated centrifuge tubes and 1 or 2 ml samples were collected. Spores were removed from diseased larvae haemolymph by centrifugation (4°C) and the supernatant stored at −18°C.

Lipid extraction

Lipids were extracted from pooled haemolymph samples (2·0 ml) by standing with 40 ml chloroform–methanol (2 : 1) overnight, in darkness, and at room temperature (FOLCH *et al.*, 1957). All operations were carried out under nitrogen when possible; all glassware and filter paper were rinsed with hot methanol. The extraction mixture was filtered (Whatman No. 1), and the residue was washed with extraction solvent. Combined washes and filtrate were washed with water (0·2 vol.) to remove non-lipid impurities and then dried and weighed as previously outlined (BENNETT and SHOTWELL, 1970).

Silicic acid column chromatography

Partially purified lipids were suspended in a minimal volume of anhydrous diethyl ether–hexane (1 : 1) and quantitatively transferred to a 1 g silicic acid column (6 × 170 mm) (BioSil HA-minus 325 mesh, Biorad Corp., Richmond, Calif.). Columns were poured as silicic acid–ether slurries and washed by gravity flow overnight with anhydrous diethyl ether. After sample application, the columns were operated under nitrogen pressure (90 mm Hg). Neutral lipids were eluted with anhydrous diethyl ether (5 ml) and phospholipids were then eluted with spectroquality methanol (5 ml). After neutral lipid and phospholipid fractions were evaporated under nitrogen and desiccated with phosphorous pentoxide, dry weights were determined. Data were obtained from 3 to 5 different haemolymph samples from healthy and diseased larvae.

Thin-layer chromatography (TLC)

Neutral lipids were separated into classes on long plates as previously described (BENNETT and SHOTWELL, 1970) with the solvent systems of FREEMAN and WEST (1966). Lipid standards were monopalmitin, cholesterol, oleic acid, 1,2- and 1,3-dipalmitin, triolein, cholesterol oleate, and methyl oleate (The Hormel Institute, Austin, Minn.). Lipid zones were detected by exposing developed plates to iodine vapours and charring with sulphuric acid.

Intact phospholipids were chromatographed on 20 × 20 cm thin-layer plates of silica gel-G (250 μ). The basic and acidic solvent systems of MAKULA and FINNERTY (1970) were used separately and in sequence to separate phospholipid moieties. Authentic standards of phosphatidic acid (PA), α-phosphatidyl glycerol (α-PG), glycerol phosphate (GP), phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), lysophosphatidyl serine (LPS), and cardiolipin (CL) (Anspec Corp., Chicago, Ill.) were used to identify haemolymph phospholipids. Zones were detected with iodine vapours, the phosphate spray of VASKOVSKY and KOTETSKY (1968), and ninhydrin (0·25% in ethanol).

Gas–liquid chromatography (GLC)

Fatty acids of neutral lipids and phospholipids were determined by GLC on polar and non-polar columns. Methyl esters were prepared after the method of Litchfield (personal communication, 1968) as outlined by BENNETT and SHOTWELL (1970). The methyl esters were evaporated under nitrogen, redissolved in 250 μl hexane, and injected (1 or 2 μl) into the preconditioned column of a Hewlett–Packard Model 810 instrument equipped with dual flame ionization detectors. Dual 4 ft × $\frac{1}{8}$ in. columns of 10% diethylenesuccinate (DEGS) on Diaport S (80–100 mesh) (Hewlett–Packard Corp., Skokie, Ill.) were temperature programmed from 100 to 225°C at 6°/min. Dual 4 ft × $\frac{1}{8}$ in. non-polar columns of 5% methyl phenyl silicone gum rubber (OV-1) on Chromasorb G (AW-DMCS) (Hewlett–Packard Corp., Skokie, Ill.) were used to separate fatty acids according to chain length. Operating parameters for both types of columns were: carrier gas (helium), 30 ml/min; hydrogen, 60 ml/min; purge air, 240 ml/min; injection port and detectors, 250°C; range and attenuation, $10^2 \times 2$ or $10^2 \times 8$. Tentative identification of fatty acids in haemolymph lipids was made by comparing their retention times to authentic standards (The Hormel Institute, Austin, Minn.) on polar and non-polar columns.

RESULTS

Lipid content of haemolymph

The amounts of neutral lipids and phospholipids (mg/ml) in haemolymph from normal and infected larvae are shown in Table 1. Total lipid content of normal larvae haemolymph ranged from 5·7 to 7·2 mg/ml. Lipid content is reduced to levels of 3·1 to 3·9 mg/ml in larvae infected for 7 days with *B. popilliae*. Both neutral lipids and phospholipids are reduced by 45 per cent as a result of milky disease. The quantity of extractable haemolymph lipids from *P. japonica* (average of three values, 6·2 mg/ml) is greater than that reported by either WYATT (1961) (3·0 mg/ml) or LUDWIG and WUGMEISTER (1953) (4·2 mg/ml). Different extraction procedures may account for these variations.

Neutral lipid classes present in healthy and diseased larvae haemolymphs are hydrocarbons (significant quantities in both healthy and diseased samples); sterol esters; triglycerides; 1,3-diglycerides and 1,2-diglycerides (both decrease in diseased

sample); free fatty acids (decreased concentration in diseased sample); cholesterol; and monoglycerides (small amounts in both samples) (Fig. 1). Classes of neutral lipids in haemolymph of *P. japonica* are similar to those found in larval stages of

TABLE 1—FRACTIONATION OF HAEMOLYMPH LIPIDS BY SILICIC ACID COLUMN CHROMATOGRAPHY*

Sample No.	Neutral lipids (mg/ml)		Phospholipids (mg/ml)	
	Healthy	Diseased	Healthy	Diseased
1	5·29	2·68	1·87	1·05
2	3·44	2·00	2·22	1·11
3	4·73	2·91	1·88	0·94
Average	4·49 ± 0·69	2·53 ± 0·35	1·99 ± 0·15	1·03 ± 0·06

Neutral lipids were eluted with diethyl ether and phospholipids were eluted with methanol.

* Recovery: 97 to 103 per cent.

Dysdercus koenigii (Hemiptera) (AGARWAL and RAO, 1969). The zones at the solvent front of TLC plates reported as unknowns are probably hydrocarbons. *P. japonica* larvae haemolymph contains two unknown lipids of low R_f value (before and after the monoglyceride zone).

Phospholipids detected in healthy and diseased larvae haemolymph are primarily phosphatidic acid, phosphatidyl choline, and phosphatidyl ethanolamine. Small quantities of glycerol phosphate and two unidentified compounds of low R_f value are present. These data correlate with those of FAST (1966) who reported that phosphatidyl choline and phosphatidyl ethanolamine are present in equal concentrations in four species of Coleoptera that he examined. All phospholipid concentrations decrease as a result of milky disease.

Quantities of fatty acids present in the neutral lipid fraction of healthy (top chart) and diseased (bottom chart) samples are compared in Fig. 2. The peaks eluting prior to and between $C_{16:0}$ and $C_{16:1}$ are not due to fatty acid methyl esters, but are probably hydrocarbons. The primary fatty acids present in larvae haemolymph are $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$. Smaller quantities of $C_{14:0}$, $C_{18:3}$, $C_{22:0}$, and $C_{22:1}$ acids are detected. Diseased samples show a reduction in all fatty acids. Composition of the phospholipid fraction is shown in Fig. 3.

Fatty acids present are, listed in order of decreasing concentrations: $C_{18:1}$, $C_{18:2}$, $C_{16:0}$, $C_{18:0}$, $C_{16:1}$, $C_{18:3}$ or $C_{20:0}$, and $C_{22:1}$. The pattern is similar to the one obtained from neutral lipids except that the relative concentration of $C_{18:2}$ is greater in the phospholipid fraction than in the neutral fraction.

Fatty acid methyl esters of neutral lipids were chromatographed on non-polar OV-1 columns (Fig. 4) with temperature programming at 6°/min from 100 to 225°C. The elution sequence of $C_{16:0}$ and $C_{16:1}$, as well as $C_{18:0}$ and the C_{18}

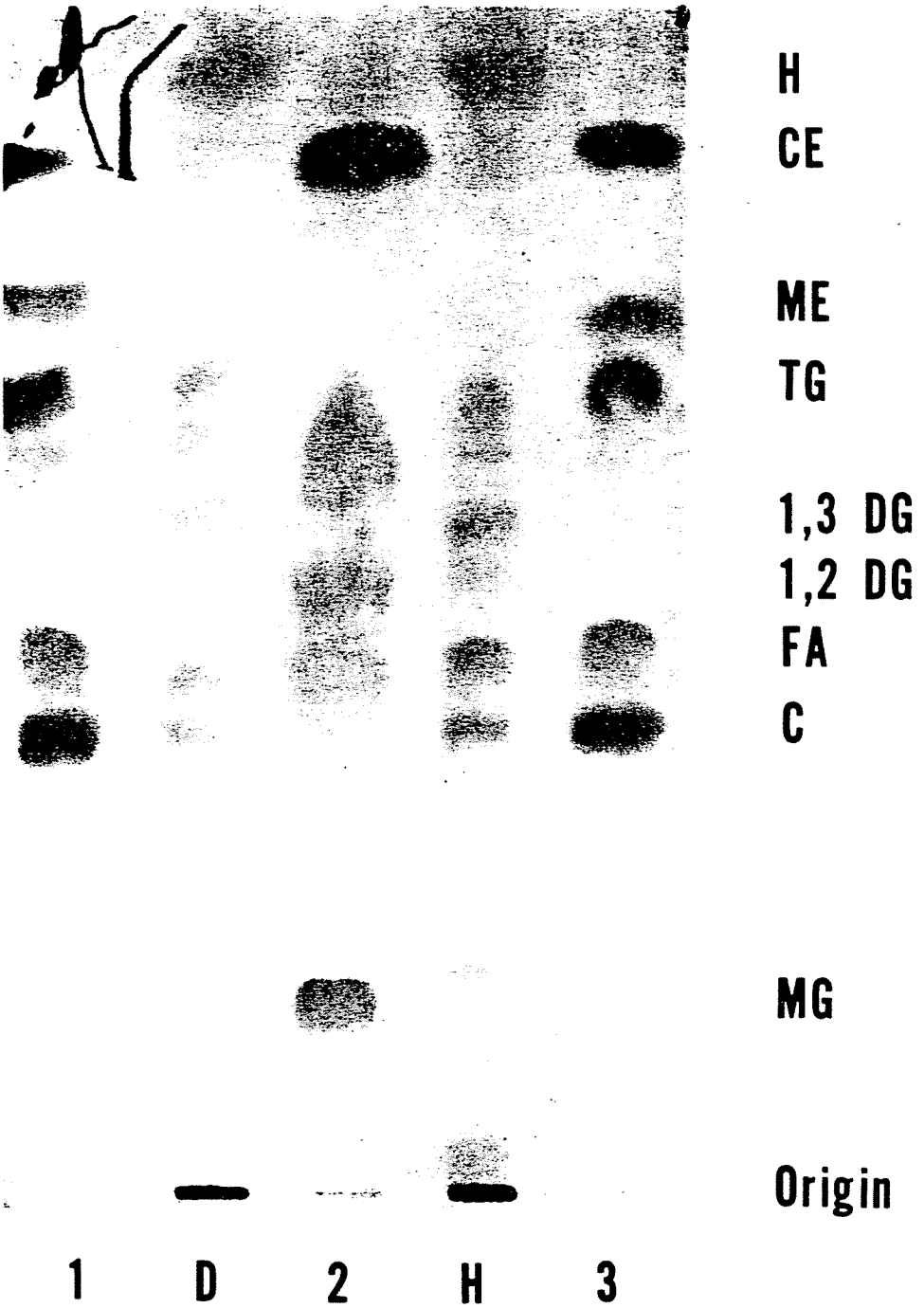

FIG. 1. Thin-layer chromatogram of healthy (H) and diseased (D) larvae haemolymph neutral lipids. 1, 2, and 3 are standard admixtures. From top to bottom: H, hydrocarbons; CE, cholesterol esters; ME, methyl esters; TG, triglycerides; 1,3- and 1,2-DG, diglycerides; FA, fatty acids; C, cholesterol; and MG, monoglycerides.

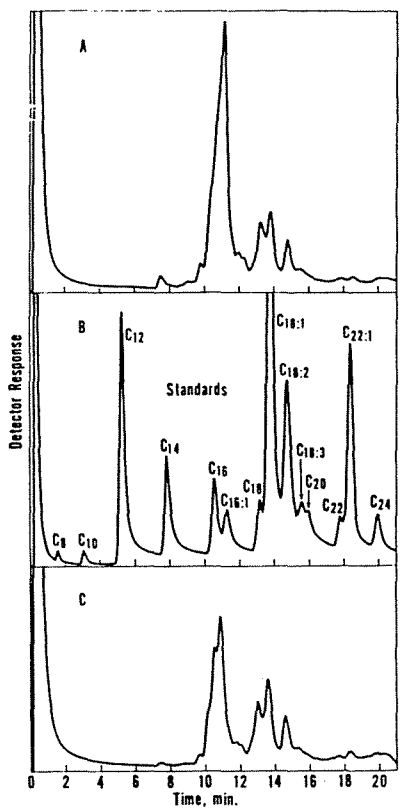

FIG. 2. Gas–liquid chromatograms of haemolymph neutral lipids from (A) healthy larvae and (C) diseased larvae on polar columns (DEGS). Fatty acid methyl esters identified by comparing retention times to authentic standards (B). Conditions described in text.

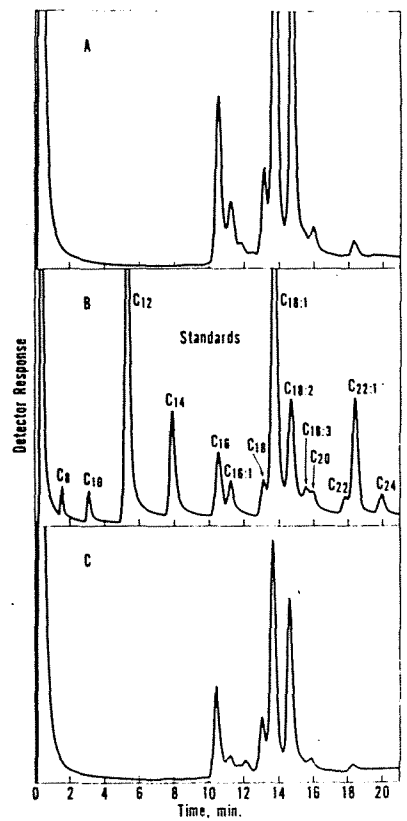

FIG. 3. Gas–liquid chromatograms of haemolymph phospholipids from (A) healthy larvae and (C) diseased larvae on polar columns (DEGS). Conditions same as for Fig. 2.

unsaturated acids, is inverted on this type of column. Compounds eluting after the C_{18} acids behave as hydrocarbons of C_{22} to C_{26} carbon atoms. Since the elution profile of the three major hydrocarbon peaks shows no change after hydrogenation or bromination, saturation is indicated. The amount of hydrocarbons present in the haemolymph is greater than the fatty acid content of the neutral lipid fraction. A complete characterization of these compounds is now being undertaken.

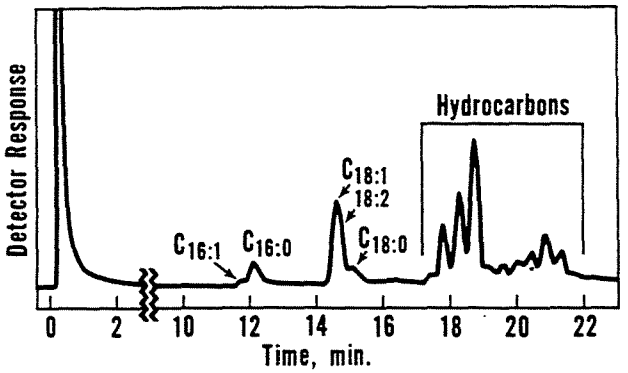

FIG. 4. Gas–liquid chromatogram of haemolymph neutral lipids on non-polar column (SE-30). Note large quantity of hydrocarbons with equivalent chain length of 22 to 26 carbon atoms. Operating conditions same as Figs. 2 and 3.

DISCUSSION

Lipid content of haemolymph from normal third instar larvae of *P. japonica* is 5·7 to 7·2 mg/ml. Lipid concentrations are similar to those reported for larval stages of other insects. FAST (1964) stated that three-quarters of the insects studied contain less than 10 per cent lipids (wet wt.). Significant variations occur in total lipids and individual lipids as insects progress through morphological stages of their life cycles (WYATT and MEYER, 1959; ACREE *et al.*, 1965; WOOD *et al.*, 1969; HUTCHINS and MARTIN, 1968). However, limited data are available concerning histopathological and chemical changes that occur in *P. japonica*, as well as in other insects, as a result of microbial invasion. The decrease of 45 per cent in the lipid content of *P. japonica* haemolymph was determined on milky larvae, but the mechanism for this non-specific reduction is not known.

Vegetative cells (*in vitro*) of *B. popilliae* have been studied for lipid content (BULLA *et al.*, 1970) and the fatty acid composition is very dissimilar to that of the larvae haemolymph. If the fatty acids of the haemolymph are utilized by the organisms, they are converted in some manner from straight-chained $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{22:1}$ acids to branched-chain C_{13}, C_{14}, C_{15}, C_{16}, and C_{17} saturated acids which make up the parasite's lipids. It appears more likely that the lipid constituents of the haemolymph alter the membrane permeability (SMITH, 1969) or influence enzyme(s) activity which permit the sporulation process to occur. Some membrane-bound enzymes require lipid cofactors for activity while

others are inhibited by lipids (SMITH, 1969; LENNARZ, 1970). A study is under way to determine the lipids of *B. popilliae* cells at different stages of milky disease. This study may indicate compositional differences resulting in the morphological differences observed in *in vivo* spores and *in vitro* spores.

Other reasons may account for the large decrease in haemolymph lipids as milky disease progresses. Infected larvae may produce enzymes that utilize lipids in efforts to remove the invading organisms. Also, pathogens may break down haemolymph lipids to simpler moieties that can be utilized as a carbon source for growth and sporulation.

The significance of the large quantities of hydrocarbons in the haemolymph is not understood. These structurally inactive compounds are certainly metabolically important in insect metabolism (ROBBINS *et al.*, 1960; LOULOUDES *et al.*, 1961). Haemolymph hydrocarbons have been reported (BAKER *et al.*, 1963; ACREE *et al.*, 1965; CHEMIK, 1969) and are similar to cuticular hydrocarbons. However, the concentrations previously reported in haemolymph from other insects are much less than those found in *P. japonica* larvae haemolymph.

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